Food and Agricultural Immunology (2002) *14*, 275–283

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Monoclonal Antibody to Fenbendazole: Utility in Residue Studies

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(Original manuscript received 26 March 2002; revised manuscript accepted 6 September 2002)

A monoclonal antibody-based ELISA was developed for fenbendazole, a widely used benzimidazole anthelmintic, with approved uses in cattle and other food animals. The antibody was elicited using as hapten 2-succinamido-5(6)-phenylthiobenzimidazole, which was conjugated with bovine serum albumin to produce an immunogen and with horseradish peroxidase to prepare a labeled ligand. The ELISA was performed on aqueous extracts of bovine liver or diluted milk samples. In bovine liver, the limit of detection was 200 ppb; in the milk matrix, the limit of detection was 3 ppb. The ELISA method is a simple approach to screen food samples for residues of fenbendazole.

Keywords: *Fenbendazole, monoclonal antibody, benzimidazole anthelmintic, drug residue*

INTRODUCTION

Benzimidazole anthelmintic drugs are widely used in both clinical medicine and veterinary practice (Campbell, 1990; Horton, 1990; Franchi *et al.*, 1999). One of these compounds, fenbendazole (FBZ; methyl 5(6)-(phenylthio)-2-benzimidazolecarbamate), is a broad-spectrum drug with approved uses in four species of food animal in the USA: cattle, swine, goats and turkeys. A related compound, febantel, is a pro-drug which is rapidly converted to FBZ *in vivo*. Regulatory tolerances for benzimidazole residues in food animal tissues have been established in the USA and abroad. The tolerance level for FBZ residues established in the USA is 0.8 ppm for the marker residue FBZ in the target tissue, liver (cattle, swine, goats), 0.6 ppm FBZ sulfoxide (also known as oxfendazole, OFZ) in bovine milk, and 6 ppm FBZ sulfone ($FBZSO_2$) in turkey liver (Code of Federal Regulations, 2001). The Joint Food and Agricultural Organization – World Health Organization Expert Committee on Food

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ISSN 0954-0105 (print)/ISSN 1465-3443 (online)/02/040275-09
DOI: 10.1080/0954010021000096364

Additives (JECFA) has recommended a single tolerance for FBZ, OFZ, and the pro-drug, febantel, of 100 ppb in milk and 500 ppb in liver (cattle, swine, sheep), expressed as the sum of FBZ, OFZ and $FBZSO_2$ (JECFA, 1996).

Methods for FBZ residue analysis in both liver and milk matrices based on chromatographic techniques have been described (Long *et al.*, 1989; Tai *et al.*, 1990; Fletouris *et al.*, 1996a,b, Capece *et al.*, 1999). These methods rely on the use of organic solvents and are excellent when quantitative analyses are required. For residue screening purposes, a qualitative test is often sufficient, especially for rapid disposition of the majority of samples lacking significant residues. We previously reported an ELISA suitable for multi-residue screening of benzimidazoles in bovine liver and milk (Brandon *et al.*, 1994, 2002) and demonstrated its utility in a regulatory setting (Brandon *et al.*, 1998). In this paper, we report an antibody more specific for FBZ and an ELISA applicable to both liver and milk matrices.

MATERIALS AND METHODS

Benzimidazoles and Related Chemicals

Fenbendazole, important metabolites, and haptens are illustrated in Figure 1. Compounds were obtained as described previously (Brandon *et al.*, 1992, 1994), except as follows. 4′-Hydroxyfenbendazole was generously provided by Professor S. A. Barker (Louisiana State University, Baton Rouge, LA, USA). The hapten used in this study (I, Figure 1) was prepared as follows.

2-Amino-5(6)-phenylthiobenzimidazole. 4-Phenylsulfinyl-*o*-phenylenediamine was prepared essentially according to the procedure of Averkin *et al.* (1975). A 7.3 g portion (33.8 mmol) was dissolved in 25 ml $CHCl_3$ and chilled in an ice-water bath. 10 ml of $CHCl_3$ solution containing 3.79 g (34.7 mmol) CNBr (97%, Aldrich, St Louis, MO, USA) was added dropwise. The solution was then stirred at room temperature for 2 h. After removal of solvent, the reaction product was taken up in ethyl acetate, sufficient KOH was added to attain pH 7.2, and the reaction product was washed with water. Ethyl acetate was removed on a rotary evaporator and the residue was redissolved in a small amount of CH_3OH. Benzene was added, CH_3OH was removed by boiling, and the solution was allowed to stand overnight at room temperature. The precipitate was filtered, washed with benzene and then ether, and air-dried to provide 7.0 g light tan solid, mp 151.0–152.0°C. MS (probe) (70 eV) m/z (relative intensity): 241 [M] + (100); 209 (10), 199 (6), 171 (8), 164 (14), 120 (13), 77 (12), 51 (15).

2-Succinamido-5(6)-phenylthiobenzimidazole. A solution of 1.53 g (6.34 mmol) of 2-amino-5(6)-phenylthiobenzimidazole and 660 mg (6.6 mmol) of succinic anhydride in 35 ml pyridine was held at room temperature for 16 h. After removal of pyridine on a rotary evaporator, the reaction residue was highly resistant to solution in either hot ethyl acetate or hot methanol. However, a warmed mixture of 50 ml CH_3OH, 10 ml water, and 0.15 ml concentrated HCl effected solution of the product. The precipitate that formed on cooling was washed sequentially with CH_3OH and ether, air-dried, and then oven-dried to give a white solid, mp 227–229°C. MS (probe) (70 eV) m/z (relative intensity): 341 [M] + (2), 324 (22), 323 (100), 268 (39), 267 (10), 241 (27), 240 (20), 159 (7).

Hapten Conjugates

The hapten was conjugated as follows, based on a procedure described by Aigner *et al.* (1982). 65 μmol of I were dissolved in 0.5 ml dimethylformamide (DMF) and 130 μmol of N-hydroxysuccinimide (in about 0.5 ml DMF) and 130 μmol of morpholinoethylisocyanide (Fluka, Ronkonkoma, NY, USA) were added and stirred for 30 min. 130 μmol of

dimethylaminopyridine were then added. 5 ml of bovine serum albumin (BSA) solution (3.6 g l^{-1} water) were then added dropwise. The solution was stirred overnight, then dialyzed against phosphate-buffered saline (PBS, 0.15 M-NaCl, 5 mM-sodium phosphate, pH 7.0). The dialyzed conjugate was analyzed by UV-visible spectrophotometry and determined to have 3.8 mol hapten/mol BSA. For preparation of the HRP conjugate, similar procedures were followed. 65 mol of hapten was reacted with 12 mg of HRP. The conjugate obtained had 4.5 mol I/mol HRP. The conjugate was stored at 1 g l^{-1} in a solution containing 10 g l^{-1} BSA and 0.2 g l^{-1} thimerosal as preservative.

Antibody Production

BALB/c mice were inoculated with the BSA conjugate of hapten and subsequently used for production of hybridomas, as described previously (Brandon *et al.*, 1994). Cultures were screened on a solid phase consisting of the same hapten conjugate. Binding to carrier protein was minimized by the presence of excess of BSA. Specificity was confirmed by ELISA using FBZ as analyte. Several positive cultures were cloned and recloned by limiting dilution and the monoclonal antibody from clone 591 (MAb 591) and its subclones was selected for further characterization. Analysis of the heavy and light chains was done using a kit (MonoAb ID HRP, Zymed Laboratories, South San Francisco, CA, USA). The MAb was determined to be IgG_1 isotype, with both κ and λ light chains. This pattern of dual light chain expression was consistently observed upon recloning of the cell line.

Competitive ELISA

Assays were conducted on polystyrene assay wells (Immulon II 96-well plates, Dynex, Chantilly, VA, USA) coated with 100 µl 5 mg l^{-1} MAb 591 IgG (4 h; all incubations conducted with shaking at room temperature, 20–25°C). Wells were washed five times with distilled water, and the remaining protein-binding sites were blocked by incubation for 1 h with a solution of 10 g l^{-1} BSA in PBS containing Tween^R-20 detergent (PBS-Tween, 0.15 M-NaCl, 5 mM-sodium phosphate, 0.05% (v/v) Tween^R-20, pH 7.0). This blocking solution (PBS-Tween + BSA) also contained 0.1 g l^{-1} thimerosal as preservative. Plates coated with IgG could be stored desiccated at 4°C for at least 6 months, provided the coated and blocked wells were incubated with 20 g l^{-1} sucrose in water for 30 min, then drained and dried at 37°C for 1 h.

Dilution series of samples were prepared in PBS-Tween + BSA, unless otherwise noted. Standard solutions of benzimidazoles were prepared as 10 ppm in DMF and stored at 4°C. Standards were diluted in PBS-Tween + BSA to 100 ppb and then in a five-fold dilution series to cover the working range of the assay (0.16 to 100 ppb). Diluted extracts or standard solutions (50 µl/well) and HRP conjugate dissolved in PBS-Tween + BSA (50 µl/well) were added sequentially to the assay wells and then mixed. After incubation for 1 h, assay wells were washed and rinsed as described above, and bound HRP conjugate was detected by reaction with substrate (6.7 mM-H_2O_2 + 1 mM-2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) in 60 mM-sodium citrate, pH 4.2) for 20–30 min (100 µl/well). The absorbance was determined at 415 nm on a microplate reader (Vmax, Molecular Devices, Sunnyvale, CA, USA). In a few experiments, tetramethylbenzidine (TMB) was used as substrate (K Blue, Neogen Corp., Lexington, KY, USA), with 0.3 N-HCl as stop solution, permitting a somewhat more sensitive assay. Readings were obtained by subtracting the absorbance at 650 nm from the absorbance at 450 nm. The results were similar to those obtained using ABTS.

ELISA Analysis of Incurred Residue Milk Samples

The study for which the milk samples were produced has been described (Barker & Kappel, 1997). In brief, FBZ was administered to three groups of ten cows (School of Veterinary

TABLE 1. Specificity of benzimidazole ELISAs using MAb 591 and MAb 587

Compound	Antibody 591 IC_{50} (ppb)	Antibody 587 IC_{50} (ppb)
Fenbendazole	2.5	3.8
Oxfendazole	420	0.62
Fenbendazole sulfone	19	8.3
4′-Hydroxyfenbendazole	16	5.3
Albendazole	140	1.4
Methyl benzimidazole carbamate	> 1000	2.4
Flubendazole	270	0.63
Oxibendazole	> 1000	1.4
Albendazole 2-amino sulfone metabolite	> 1000	> 1000
Thiabendazole	> 1000	> 1000

Medicine, Louisiana State University, Baton Rouge, LA, USA). The drug was administered as a feed top dressing, a drench, or as a paste, with a target dose of 5.0 mg kg^{-1}. Milk samples were collected at the time of dosing (0 h) and at 12-h intervals for 72 h and were frozen until analyzed by LC (Long *et al.*, 1989). Samples were refrozen and maintained frozen until analyzed by ELISA at the Agricultural Research Service, Albany, CA, USA. Milk samples were thawed at room temperature and mixed by shaking immediately before analysis. Serial dilutions (1:2 to 1:256) were prepared using a Biomek 1000 workstation (Beckman Instruments, Fullerton, CA, USA). A FBZ standard was also diluted robotically.

ELISA Analysis of Liver Samples

Fortified liver samples and one liver sample containing incurred FBZ residues (#281, described by Wilson *et al.*, 1991) were prepared and extracted with water as described previously (Brandon *et al.*, 1994, 1998).

RESULTS AND DISCUSSION

ELISA

A typical standard curve for the ELISA using antibody 591 is shown in Figure 2. The working range, defined operationally as the 20% inhibitory concentration (IC_{20}) to the IC_{80} was approximately 0.8 to 10 ppb. A comparison between specificities of this antibody with the previously reported MAb 587 is shown in Table 1. MAb 591 recognizes the 5-substituent of FBZ, although there is significant binding to the sulfone metabolite. In contrast, MAb 587, elicited using hapten II (Figure 1), recognizes common features of the methyl benzimidazole-carbamates, with reduced binding to the bulkier substituents of $FBZSO_2$ and 4′-OH-FBZ.

Limits of Detection and Recovery of FBZ

The lower limit of detection, defined by the 95% one-sided lower confidence limit, was determined for FBZ. Whole milk samples were spiked with known amounts of analyte then diluted for analysis. Table 2 shows that the assay detected less than 10 ppb with high recovery. Qualitative results could be obtained without the aid of a microplate reader, with a lower limit of detection of about 20 ppb FBZ. In the liver matrix, the lower limit of detection, with water extraction, was 200 ppb for extracts analyzed neat, or 550 ppb when extracts were analyzed after a five-fold dilution.

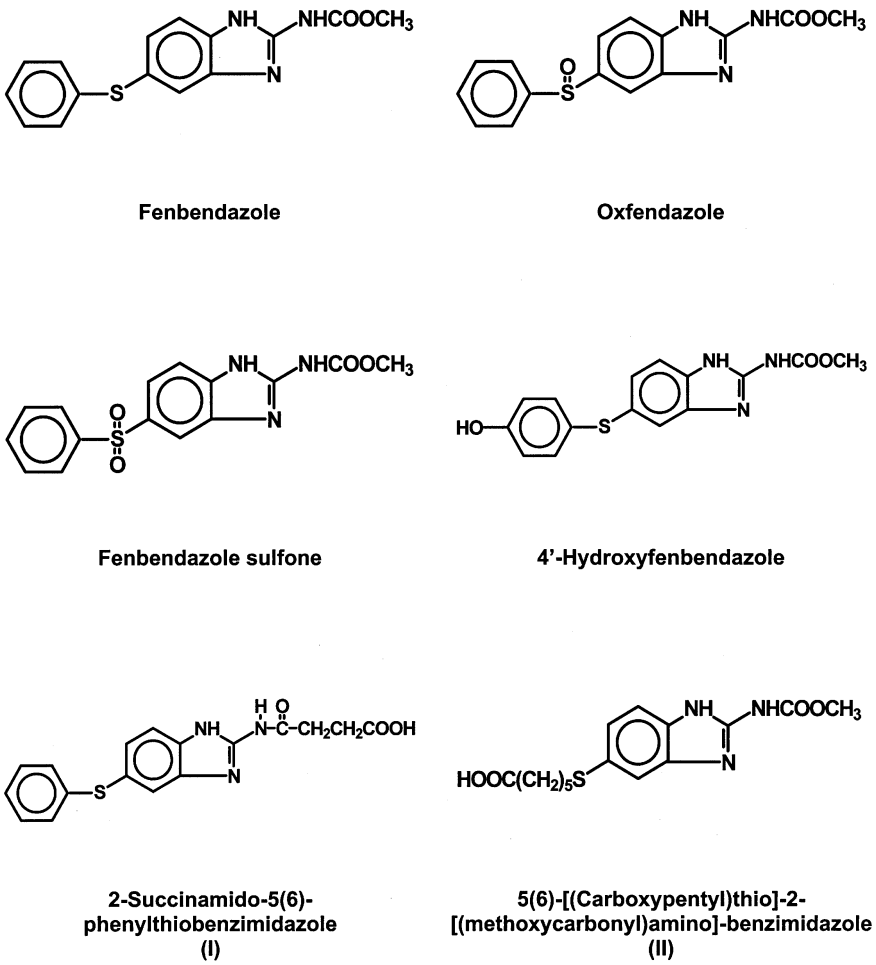

FIG. 1. Fenbendazole, oxidized metabolites, and haptens used to elicit antibodies.

Matrix Effects

To determine whether milk or liver extracts interfered with the assay, FBZ standards were prepared, diluted in buffer, raw milk, homogenized milk (neat and 1:5), nonfat milk, or liver extract (1:5). Standard curves were obtained. Raw milk and homogenized milk produced similar curves, and nonfat milk gave results equivalent to buffer. It therefore appeared that the

TABLE 2. Limits of detection and recovery of FBZ in milk[a]

Substrate	Lower limit of detection (ppb)	% Recovery
ABTS	3	95 ± 25
TMB	2	99 ± 11

[a] *Recoveries determined in the range 10–100 ppb for FBZ.*

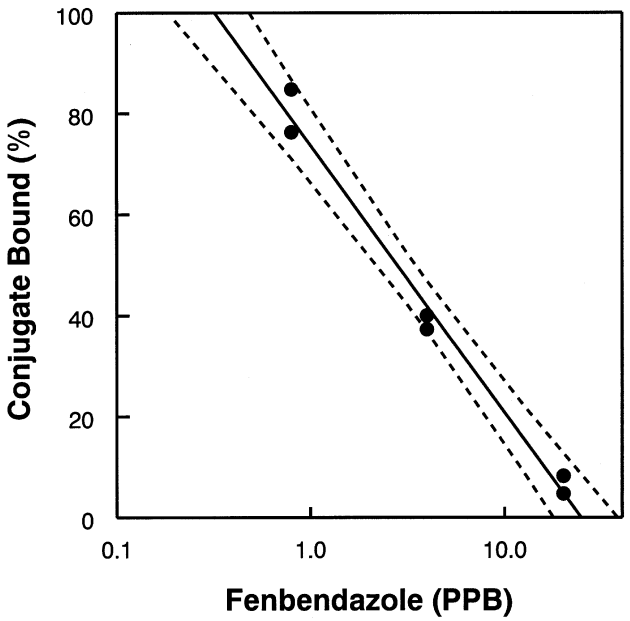

FIG. 2. Linear portion of standard curve for ELISA with monoclonal antibody 591, with 95% confidence interval.

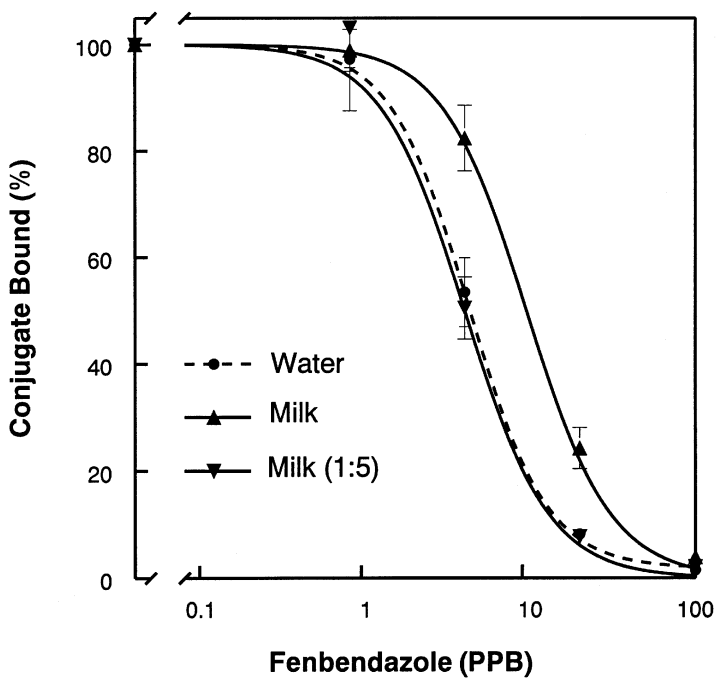

FIG. 3. Milk matrix effects: standards were diluted in water, milk (neat and 1:5).

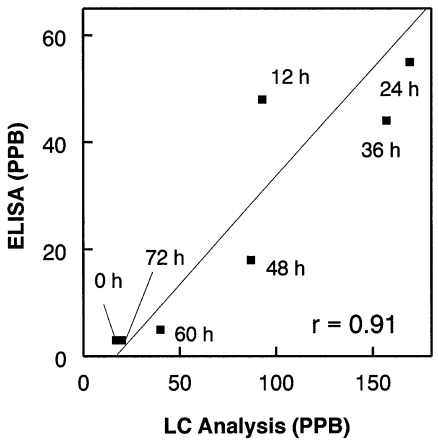

FIG. 4. Correlation of LC and ELISA data. Total residues from the LC data for cows treated by FBZ drench were compared to the ELISA results. Data for 10 animals were averaged for each of the 7 time points (0 to 72 h after dosing).

fat might be responsible for the matrix effects. Representative data for milk are shown in Figure 3. Dilution of milk or liver samples at least five-fold was sufficient to eliminate significant matrix effects in the assay, as indicated by changes in IC_{50} or slope. The matrix effects would only be important if quantification of FBZ were desired below 10 ppb.

Analysis of Incurred Milk Samples From FBZ-treated Cattle

To compare the ELISA to LC analysis for incurred milk samples, data for each time point (0 to 72 h) were averaged for the 10 animals treated with FBZ drench. Figure 4 shows that there was a good correlation between estimates of FBZ in milk obtained by ELISA and total FBZ residues estimated by LC ($r = 0.91$). Table 3 shows the result of analysis of 207 milk samples, with suggested criteria for use of the FBZ ELISA. The data indicate low levels of false negatives ($< 3\%$) and modest levels of false positives compared to LC analyses ($< 14\%$).

Analysis of Fortified and Incurred Liver Samples

A panel of 46 samples were prepared by one member of the team and were analyzed 'blind' by a second member. The results indicated that eight samples containing FBZ at 400 ppb or higher were scored as positive. Thirty-eight samples were scored as negative, including eight blanks, one sample containing 50 ppb FBZ, and 29 samples fortified with other

TABLE 3. Specificity and sensitivity of ELISA of FBZ in milk

	Analytical criterion by LC (ppb)[a]		
ELISA	FBZ + FBZSO + FBZSO2 ≥ 0	FBZ + FBZSO + FBZSO2 ≥ 100	FBZSO ≥ 100
Criterion for positive ELISA	> 6 ppb	> 6 ppb	> 25 ppb
% False negative	1.0	1.0	2.8
% False positive	3.3	14	12

[a] $n = 207$.

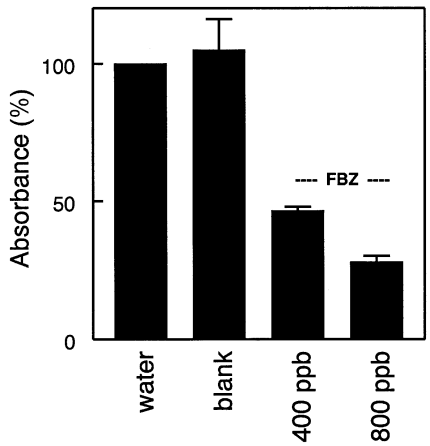

FIG. 5. ELISA analysis of negative control liver samples and samples fortified with 400 or 800 ppb FBZ. The bars indicate the relative yellow color (A_{450} - A_{650}), after TMB substrate development was stopped by acidification, as a percent of the water control sample.

benzimidazole derivatives. It should be noted that the positive samples could readily be distinguished from negative control liver samples by visual inspection of the developed ELISA plate (see Figure 5). A bovine liver sample containing incurred residues of FBZ was analyzed by ELISA following aqueous extraction and determined to contain 1170 ppb FBZ. When dried ethyl acetate extracts of this sample were redissolved in methanol, diluted in water, and analyzed by ELISA, a value of 970 ppb was obtained. This sample had previously been found to contain 1040 ppb by LC analysis following extraction with ethyl acetate (Wilson *et al.*, 1991).

CONCLUSIONS

The MAb-based ELISA described in this report provides a simple analytical method for FBZ and could be used to measure residues in milk and liver. For milk, no sample preparation is involved. In its present form, the test requires minimal equipment, and samples with FBZ residues as low as 20 ppb can be identified as positive by eye. The sensitivity could be adjusted by use of a suitable dilution to achieve the desired cutoff. Both the study of [14-C]-FBZ depletion (Kappel & Barker, 1996) and the LC study of FBZ residues (Barker & Kappel, 1997) concluded that residues of FBZ become undetectable close to 72 h. The present ELISA results are in agreement (Figure 4). For analysis of bovine liver samples, aqueous extraction appeared sufficient for a simple screen, with sensitivity at half the US tolerance level of 800 ppb.

ACKNOWLEDGEMENTS

The authors thank Drs Rainer K. Muser and Larry Deetz (American Hoechst Corporation) and Professor Steven A. Barker (Louisiana State University) for their cooperation in making samples available for this study.

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